



## Internal bacterial contamination of micropropagated hazelnut: identification and antibiotic treatment

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### Introduction

Commercial micropropagation laboratories very often report that persistent bacterial and fungal contamination is a serious problem [1–3]. Failure of surface sterilization procedures to produce aseptic cultures is a problem especially with woody plants. Isolated meristems [4] and explants from stock plants grown under controlled conditions [5] have been used to obtain aseptic cultures for some plants. Contamination is not always seen at the culture establishment stage; some internal contaminants become evident at later subcultures and are difficult to eliminate [6]. Detection at an early stage can aid in selecting bacteria-free cultures [7]. Antibiotic or other treatments may be needed to eliminate persistent microbial contamination [8–11], but the type and level of antibiotics and the duration of treatment useful for different plant tissue cultures vary and therefore need to be determined before use [11,12].

Internal bacterial contamination was observed in hazelnut shoot cultures in our laboratory. Contaminants were evident at culture establishment or became apparent after several subcultures. Loss of plants resulted when bacteria overgrew plant material but some explants survived and continued to grow with bacteria present. In this study we isolated bacteria from hazelnut shoot cultures, characterized and identified them, and determined the effects of antibiotic treatments on bacteria and plant materials.

### Procedure

#### Plant material

Shoot cultures from infected hazelnut cultures (*Corylus avellana* L., *C. contorta*, C.) were from the USDA-ARS National Clonal Germplasm Repository (NCGR) collections: 'Tonda Gentile Romana' (Cor 5), 'Hall's

Giant' (Cor 16), 'Cutleaf' (Cor 18), *C. contorta* (Cor 50), 'OSU 20-58' (Cor 79), 'Giresun 54–60' (Cor 96), 'Fitzgerald' (Cor 105), *C. avellana* (Cor 187), 'Bergieri' (Cor 262), 'Badem' (Cor 415), and 'Cosford Sel 3L' (Cor 494).

#### Detection and isolation of bacteria

Segments of tissue cultured plantlets were inoculated into a liquid nutrient broth containing 0.8% nutrient broth (Difco; Sigma Chemical Co., St. Louis, MO) with 1% glucose and 0.5% yeast extract at pH 6.9 and incubated at room temperature until visibly turbid. The bacteria were then streaked onto nutrient agar plates and purified by repeated streaking.

#### Identification of bacterial isolates

Standard bacteriological tests were performed on the cultures and on known standard organisms. Results of these tests and colony morphology were used to classify the bacteria. Gram-stain, oxidase, oxidative/fermentive (O/F), starch hydrolysis (SH), motility and gelatinase tests [13] and colony description were performed on isolates. Three-day-old bacterial cultures (7-day for two slow-growing ones) were used for these tests. Comparisons were made with descriptions in Bergey's Manual of Determinative Bacteriology [14]. Some cultures were tested for carbon metabolism using the Biolog system and the following procedure. Cultures were grown for 24 h on tryptic soy agar (Sigma) without a carbon source. Cultures were inoculated into physiological saline solution to a predetermined turbidity and pipetted onto Biolog plates containing 96 different carbon sources. Plates were read manually at 4 and 24 h and at 48 h for some slow growing cultures. Results were compared to the Biolog database for identification.

### *Effect of antibiotics on bacterial isolates*

Minimal inhibitory concentrations of the antibiotics were found by using a tube dilution method for standard bacterial cultures and for bacterial isolates from the infected hazelnut plants. Tubes were inoculated with one drop of bacteria from 3- to 4-day-old cultures into liquid MS medium [15] containing 2 ml of the following concentrations. For single antibiotics, streptomycin sulfate or Timentin at 62.5, 125, 250, 500 or 1000  $\mu\text{g ml}^{-1}$  or gentamicin at 6.25, 12.5, 25 or 50  $\mu\text{g ml}^{-1}$ . For combinations of antibiotics, Timentin + streptomycin at 125+250, 125+500, 250+250 or 250+500  $\mu\text{g ml}^{-1}$ ; Timentin + gentamicin at 125+6.25, 125+12.5, 250+6.25 or 250+12.5  $\mu\text{g ml}^{-1}$ ; gentamicin + streptomycin at 6.25+250, 6.25+500, 12.5+250, 12.5+500  $\mu\text{g ml}^{-1}$ . Effectiveness was determined by putting a drop of each culture onto sections of nutrient agar plates and checking for growth after 4 to 6 days.

### *Antibiotic treatment of plant material*

Timentin (500  $\mu\text{g ml}^{-1}$ ) + streptomycin sulfate (1000  $\mu\text{g ml}^{-1}$ ) or gentamicin (12.5  $\mu\text{g ml}^{-1}$ ) + streptomycin (1000  $\mu\text{g ml}^{-1}$ ) were used in liquid MS to treat *C. avellana* cv. 'OSU 20-58'. Shoot tips (1 cm) and first node cuttings (0.5 cm) from six plantlets were submerged into individual tubes containing 3 ml of the two treatments for 6 days. After 6 days, the condition of the plant material was noted, and liquid was removed from the tubes so that only the bases were submerged for the remaining 4 days. Controls (plant tissues grown in liquid MS without antibiotics) were also included with each experiment. After antibiotic treatment (10 days), the plant condition was again noted and the plants were placed in individual tubes of semi-solid *Corylus* multiplication medium. The bases were placed into nutrient broth for detection of bacterial growth. At the next transfer (3 weeks), the plant bases were streaked onto a bacterial detection medium, 523 agar plates [16], before transfer to new medium. The bases were streaked on 523 medium on all subsequent transfers.

The second experiment treated three genotypes with two antibiotic combinations. 'Hall's Giant', 'OSU 20-58', and 'Giresun 54-60' were treated as above with Timentin (500  $\mu\text{g ml}^{-1}$  or 1000  $\mu\text{g ml}^{-1}$ ) + streptomycin sulfate (1000  $\mu\text{g ml}^{-1}$ ).

Phytotoxicity of antibiotics was determined visually by checking for browning, chlorosis, and morphological changes. All shoot cultures were kept at 25 °C

and 16-h photoperiod (25  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Tests for additional treatments followed this same procedure.

## **Results and discussion**

### *Identification of bacterial isolates*

Colonies were visible on nutrient agar plates in 3 days for most bacteria but some were slow-growing and required 7 days for colonies to be visible. Colony pigmentation varied from white and beige to yellow and pink to pink-red. The results of oxidase, starch hydrolysis, oxidative/fermentive, motility, and gelatinase tests varied with the isolates. Isolates identified included *Agrobacterium radiobacter* B, *Pseudomonas fluorescens*, *Xanthomonas* spp., *Enterobacter asburiae*, *Flavobacterium* spp. and *Alcaligenes* spp. Many isolates were not identified and were not similar to bacteria in the Biolog database.

### *Effect of antibiotics on bacterial isolates*

Initial experiments showed that streptomycin and Timentin were ineffective on most of the bacterial isolates. Gentamicin was the most effective, controlling approximately half of the isolates including those from Cor 96 and Cor 415b (*Enterobacter* spp.) at concentrations as low as 6.25  $\mu\text{g ml}^{-1}$ . No single antibiotic was effective for all bacterial isolates from hazelnut shoot cultures. *Alcaligenes* and eight others were not inhibited by any single treatment. When gentamicin and Timentin were effective, it was usually at a very low concentration. Streptomycin often required the highest concentration for effective treatment. Young et al. [17] also reported that among rifampicin, tetracycline, cefotaxime, and polymyxin B, no single antibiotic was bactericidal against all of the bacterial isolates from shoot cultures of several woody plants, but each of the isolates was killed by at least one of the antibiotics.

Broader testing with Timentin, gentamicin, and streptomycin showed that combinations of the antibiotics were more effective for killing bacteria than single antibiotics. Streptomycin combined with Timentin or gentamicin was effective in killing all of the bacteria tested. Timentin and gentamicin combined were effective with most isolates. Combinations of all three antibiotics were also effective.

Table 1. Percentage of bacteria-free hazelnut plantlets produced from contaminated shoot tips treated with two concentrations of combinations of Timentin (T) and streptomycin (S) for 10 days.

Plants treated	Bacteria	Bacteria-free plants/treated plants	
		T(500)+S(1000) <sup>a</sup>	T(1000)+S(1000)
'Hall's Giant'	Gram negative	5/10	7/10
'OSU 20-58'	<i>Alcaligenes</i>	6/10	9/10
'Giresun 54-60'	<i>Enterobacter</i>	6/10 <sup>b</sup>	na

<sup>a</sup>Antibiotic treatment ( $\mu\text{g ml}^{-1}$ ).

<sup>b</sup>Six were bacteria free, but three died after treatment was completed.

na, data not available for this treatment.

### Antibiotic treatment of plant materials

Initial tests showed that a 10 day treatment with a combination of streptomycin and Timentin ( $250 \mu\text{g}$  each) was ineffective for eliminating unidentified Gram-negative bacteria from 'Tonda Gentile Romana', and 'Hall's Giant', or *Alcaligenes* from 'OSU 20-58'. Initially some cultures appeared bacteria free, but later indexing showed bacterial growth. 'OSU 20-58' shoot tips treated for 10 days with Timentin ( $500 \mu\text{g ml}^{-1}$ ) + streptomycin ( $1000 \mu\text{g ml}^{-1}$ ); or gentamicin ( $12.5 \mu\text{g ml}^{-1}$ ) + streptomycin ( $1000 \mu\text{g ml}^{-1}$ ) produced 12–25% bacteria-free cultures.

'Hall's Giant', 'OSU 20-58', and 'Giresun 54-60' treated with Timentin ( $500 \mu\text{g ml}^{-1}$  or  $1000 \mu\text{g ml}^{-1}$ ) + streptomycin ( $1000 \mu\text{g ml}^{-1}$ ) resulted in more bacteria-free cultures than the earlier tests (Table 1). Treatments of 'Giresun 54-60' infected with *Enterobacter* produced 60% bacteria-free shoots, but half of them died due to the phytotoxicity of the treatment. Repeat tests are in progress.

Combinations of antibiotics are used against bacteria from plant tissue cultures [10,17]. Young et al. [17] used a combination of  $25 \mu\text{g}$  cefotaxime,  $25 \mu\text{g}$  tetracycline,  $6 \mu\text{g}$  rifampicin, and  $6 \mu\text{g ml}^{-1}$  polymyxin B to treat bacteria in tissue cultures of apple, *Rhododendron*, and Douglas-fir but later found that the bacteria were still present. Leifert et al. [10] reported that a range of different bacteria were eliminated from contaminated plant tissues of *Hemerocallis*, *Choisya* and *Delphinium* using combinations of gentamicin, streptomycin, carbenicillin, cephalothin and rifampicin.

Hazelnut shoot tips showed some antibiotic phytotoxicity but severe damage was evident in nodal cuttings. The combination of gentamicin and streptomycin produced the greatest phytotoxicity.

Both gentamicin and streptomycin belong to the group of aminoglycoside antibiotics. They bind to 30S ribosomal subunits in bacterial cells and inhibit olein synthesis and may also inhibit protein synthesis in chloroplasts and mitochondria in plant tissues [17], therefore resulting in small and yellow leaves. Phytotoxicity to gentamicin was shown in *Mentha* [12] and to cell growth of *Helianthus tuberosus* by streptomycin [11] and to shoot cultures of *Clematis*, *Delphinium*, *Hosta*, *Iris* and *Photinia* by streptomycin [18]. Gentamicin  $50 \mu\text{g ml}^{-1}$  was added to pear culture medium without harm to the plants [8]. Streptomycin was effective in eliminating bacteria from infected mint cultures with little phytotoxicity [7].

### Conclusions

Internal bacterial contaminants in tissue cultured hazelnuts were eliminated by antibiotic treatment. Single antibiotics were ineffective, but combinations of two or more eliminated most contaminants. Streptomycin combined with Timentin or gentamicin killed all of the isolated bacteria tested, as did a combination of all three. Timentin combined with gentamicin was effective for most isolates. In plant tissues, antibiotic concentrations 3–4 times higher than those effective on isolated bacteria were needed to eliminate internal bacteria. Combinations of streptomycin with gentamicin and streptomycin with Timentin were effective in eliminating persistent bacterial contamination in hazelnut plants. Phytotoxicity varied with antibiotic type and plant genotype.

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